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Common genetic variants of *TP53* and *BRCA2* in esophageal cancer patients and healthy individuals from low and high risk areas of northern China

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Abstract

TP53 and *BRCA2* are frequently mutated in cancer and polymorphisms of these genes may modify cancer risk. We used SSCP and DNA sequencing to assess and compare frequencies of R72P (*TP53*) and 5′UTR203G>A, N372H, and K1132K (*BRCA2*) polymorphisms in healthy Chinese subjects at varying risk for esophageal squamous cell carcinoma (ESCC) and in ESCC patients. Suggestive overall differences in the distributions of genotypes by risk groups were seen for all genotypes except K1132K. Differences in R72P and N372H were most likely a reflection of lack of Hardy–Weinberg equilibrium (HWE), however, the difference in 203G>A was due to low prevalence of GG in ESCC patients (0.22 versus 0.36 in high risk group ($P = 0.047$), and 0.22 versus 0.40 in low risk group ($P = 0.010$)), consistent with a disease association. These data suggest that the 203G>A polymorphism in *BRCA2* may be associated with risk of ESCC.
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Keywords: Esophageal cancer; *TP53*; *BRCA2*; Polymorphisms; Cancer risk; China

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common fatal cancers worldwide. There is great geographic variation in the occurrence of this tumor, including Shanxi Province, a region in north central China with some of the highest esophageal cancer rates in the world [1–4]. While epidemiologic studies indicate that tobacco and alcohol are the major risk factors for esophageal cancer in the low risk regions of Europe and North America, the etiology in high risk areas remains less clear. Several possibilities, including nitrosamines, nutritional deficiencies, fermented and moldy foods, exposure to polycyclic aromatic hydrocarbons, and infectious agents such as human papillomavirus (HPV) have been considered, but none has been convincingly linked to Shanxi's high rates of esophageal cancer [5,6].

It is well known that mutations in *TP53* are the most common genetic alterations in human cancer. *BRCA2*, another gene frequently mutated in cancer, was originally identified by its association with an increased risk of breast cancer [7]. Both the *TP53* and *BRCA2* genes have been implicated in cell cycle control [8,9]. Esophageal cancer is a very common disease in many areas of China, especially in Shanxi Province [10]. In our previous studies in ESCC

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patients from Shanxi, high frequencies of allelic loss were found on chromosome 17p13 where *TP53* is located, and on chromosome 13q12 where *BRCA2* is located [11–13]. Further analyses have shown that these ESCC patients have frequent mutations (77% for *TP53* and 9% for *BRCA2*) as well as frequent allelic loss at polymorphic sites within these genes: 73% had a lost allele at R72P in *TP53*, and 46% had lost at least one allele from 1 of 3 polymorphisms, 5′UTR203G>A, N372H, and K1132K) in *BRCA2* [14,15]. These results suggest that both *TP53* and *BRCA2* are involved in ESCC tumorigenesis and advance the notion that genetic polymorphisms in these genes may play a role in individual susceptibility to esophageal cancer.

Polymorphisms in the *TP53* and *BRCA2* genes have previously been studied as modifiers of cancer risk. The wildtype *TP53* gene shows a polymorphism at codon 72 that involves a single base change and results in an Arg or R (CGC) to Pro or P (CCC) amino acid replacement in the transactivation domain of the protein (Arg72Pro or R72P). Although some studies have reported an association between the PP genotype and increased risk for cancer in high risk subgroups [16], other studies have not [17]. Recently, Healey et al. reported that a common polymorphism in exon 10 of *BRCA2* (N372H) conferred increased risk of breast cancer: those with the HH genotype had a 1.31-fold greater risk than those with the NN genotype [18]. From our previous studies it appears that several of these polymorphisms exist in ESCC patients and one of the alleles is frequently lost [14,15]. However, the distributions of polymorphisms in *TP53* and *BRCA2* are poorly defined in the Chinese population, especially northern Chinese. There have been just two reports to date in the literature relating the R72P polymorphism in *TP53* to ESCC, a modest sized study from Taiwan [19] and a smaller study from Linxian in northern China [20]. There are no reports of the association between *BRCA2* polymorphisms and ESCC.

The primary aim of this study was to assess and compare the frequencies of selected known polymorphisms in *TP53* and *BRCA2* in Chinese subjects at varying degrees of risk (i.e. healthy individuals from low and high risk areas in northern China, and ESCC patients). We also sought to determine whether three germline variants of *BRCA2* reported in our previous study [15] are novel polymorphisms or true germline mutations in this population. Altogether we examined a total of seven genetic variants, including 1 in *TP53* and six in *BRCA2*, by using single strand conformation polymorphism (SSCP) analyses and DNA sequencing.

2. Materials and methods

2.1. Healthy individuals from low/high risk areas

Two groups of healthy individuals were selected as follows: the low risk group included 101 individuals (males = 56, females = 45) from Beijing where crude esophageal cancer mortality rates are 18.2/100,000 in males and 8.2/100,000 in females [10]; and the high risk group was comprised of 131 healthy individuals (males = 80, females = 51) from Yangcheng county, which has one of the highest rates of esophageal cancer in China (crude esophageal cancer mortality rates of 167.9/100,000 in males and 94.9/100,000 in females, [10]). The age range for both groups was 35–55 years.

2.2. ESCC patient selection from hospital

Patients presenting in 1995 and 1996 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, who were diagnosed with ESCC and considered candidates for curative surgical resection, were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the US National Cancer Institute. A total of 120 patients (74 male and 46 female) with ESCC were selected who had a histologic diagnosis of esophageal squamous cell cancer confirmed by pathologists at both the Shanxi Cancer Hospital and the NCI. None of the patients had prior therapy, and Shanxi was the ancestral home for all. Out of the120 ESCC patients, 56 had previously been examined for genetic alterations in *TP53* and *BRCA2* [14,15], while the remaining 64 had not been examined for these two genes. The patients ranged in age from 35 to 75 years, and 41% had a family history of upper gastrointestinal (UGI) cancer (including 1st, 2nd and 3rd degree relatives).

2.3. Biologic specimen collection and processing

Ten milliliter of venous blood was taken from each patient prior to surgery. Blood from healthy individuals was collected from blood banks in Beijing and Yangcheng. Genomic DNA was extracted and purified using standard methods.

2.4. PCR–SSCP analysis

Seven pairs of primers were used in this study (Table 1). R72P in *TP53*; and 203G>A, N372H, and K1132K in *BRCA2* are all previously reported polymorphisms (http://www.iarc.fr/p53/Poly.HTM,) (http:/www.nhgri.nih.gov/Intramural_research/Lab_transfer/BIC). R118H, C315S, and P3300S in *BRCA2* are germline alterations found in our previous study [15]. PCR reactions and SSCP were carried out using the methods previously described [14,15]. Examples of SSCP are shown in Fig. 1.

2.5. DNA sequencing

Several different migrating patterns in *TP53* and *BRCA2* were observed in the three different groups of subjects studied. All bands shifted from the normal control were excised and the DNA sequenced using methods described previously [14].

Table 1
Primer sequences of *TP53* and *BRCA2* variants used in PCR-SSCP analyses

Primer name	Sequences		Size of PCR product (bp)	Variant		
	Sense primer (5′–3′)	Antisense primer (5′–3′)		Gene/exon/codon	Base change	Amino acid substitution
R72P	TCCTCTGACTGCTCTTTTC	GAAGGGACAGAAGATGACAG	220	*TP53*/exon 4/72	CGC → CCC	Arg → Pro
203G>A	CTCAGTCACATAATAAGGAATGC	CAACACTGTGACGTACTGGGT	258	*BRCA2*/NT203	G → A	–
R118H	ACACTTCCAAAGAATGCAAAT	TCTTCCTACAGGCTCTTAG	296	*BRCA2*/exon 4/118	CGC → CAC	Arg → His
C315S	CAAAGACCACATTGGAAAGTC	GATCAGTATCATTTGGTTCCAC	259	*BRCA2*/exon 10/315	TGT → AGT	Cys → Ser
N372H	AAGCAAACGCTGATGAATGTG	TGGTCACATGAAGAAATATGC	260	*BRCA2*/exon 10/372	AAT → CAT	Asn → His
K1132K	GTCATATAACCCCTCAGATG	CTGTACCTTCAAATTGCTTGC	306	*BRCA2*/exon 11/1132	AAA → AAG	Lys → Lys
P3300S	AGAGAAGAGCCTTGGATTTCT	TGGGTATTTATCAATGCAAGT	250	*BRCA2*/exon 27/3300	CCA → TCA	Pro → Ser

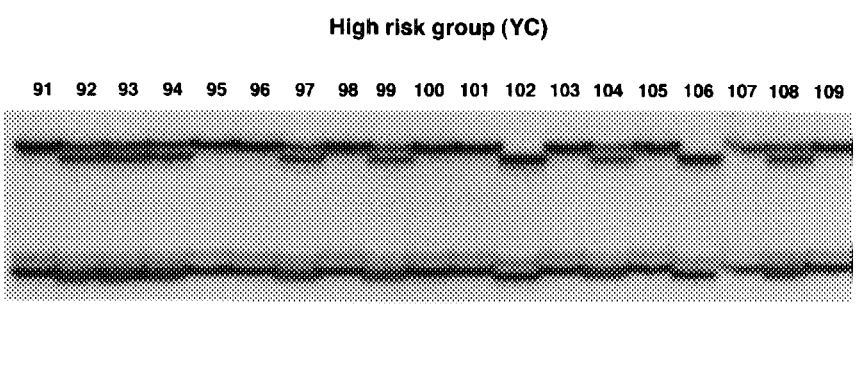

Fig. 1. SSCP for 203G>A (primer exon 2) of *BRCA2* in ESCC.

2.6. Statistical analysis

All statistical analyses were performed using Statistical Analysis Systems (SAS) (SAS Corp., NC). Hardy–Weinberg equilibrium (HWE) was assessed within each risk group by comparing the observed numbers of different genotypes with those expected under HWE for the distribution of genotypes and estimated allele frequencies using χ^2 tests with 2 or 1 d.f., respectively. We examined overall differences between risk groups (low risk group (healthy Beijing subjects), high risk group (healthy Yangcheng subjects), and ESCC patients, and the various genotypes (generically called wildtype/wildtype (WW), wildtype/variant (WV), and variant/variant (VV)) using 3 × 3 contingency tables and χ^2 tests. For each genotype that showed an overall difference with $P < 0.10$ in our 3 × 3 tables, we evaluated 2 × 3 tables to compare the risk groups (i.e. low versus high risk groups, low risk group versus ESCC, and high risk group versus ESCC) by genotypes. We also tested for a monotonic increase in gene frequency (0 = WW, 1 = WV, 2 = VV) with increasing risk group (0 = low risk group, 1 = high risk group, and 2 = ESCC) with ordinal proportional odds regression models (using SAS, PROC CATMOD). A likelihood ratio test for trend which treated risk group as a continuous variable was used to test whether there was an association between increasing risk group and increasing variant genotype (χ^2 with 1 d.f.). No differences in the distribution of genotypes by gender were seen when examined within risk groups or overall (data not shown), so only data for combined genders are presented here.

3. Results

We studied a total of 352 individuals from three different risk groups: 101 healthy individuals from a low risk area (Beijing); 131 healthy individuals from a high risk area (Yangcheng); and 120 patients with ESCC (Taiyuan). The distributions of allele frequencies for the seven germline alterations evaluated in the three risk groups are shown in Table 2. Also, three novel variants (P36P, P47P and G59A) near R72P in exon 4 of *TP53*, and 2 variants (K115E and

Table 2
Allele frequencies for selected genetic alterations by risk group

Risk group	*TP53* alteration (alleles) R72P (R/P)	*BRCA2* alterations (alleles)					
		203 G>A (G/A)	R118H (R/H)	C351S (C/S)	N372H (N/H)	K1132K (K/K)	P3300S (P/S)
Low risk group (Beijing)							
Male, 56; female, 45	0.52/0.48[a]	0.64/0.36	1.00/0.00	1.00/0.00	0.75/0.25	0.62/0.38[b]	1.00/0.00
High risk group (Yangcheng)							
Male, 79; female, 51	0.55/0.45[c]	0.63/0.37	1.00/0.00	0.99/<0.01	0.75/0.25	0.63/0.37[d]	0.99/<0.01
ESCC cases							
Male, 74; female, 46	0.48/0.52[e,f]	0.55/0.45	1.00/0.00[g]	1.00/0.00	0.74/0.26	0.61/0.39	1.00/0.00

[a] Two persons in the low risk group also had a P36P alteration identified while testing for R72P.
[b] Two persons in the low risk group also had a S1140S alteration identified while testing for K1132K (variant not in BIC database).
[c] Two persons in the high risk group also had a G59A alteration identified while testing for R72P (variant not in IARC *TP53* database).
[d] One person in the high risk group also had a S1140S alteration identified while testing for K1132K (variant not in BIC database).
[e] One persons in the ESCC group also had a P36P alteration identified while testing for R72P.
[f] One person in the ESCC group also had a P47P alteration identified while testing for R72P.
[g] One person in the ESCC group also had a K115E alteration identified while testing for R118H.

Table 3
Genotype frequencies for 1 *TP53* and three *BRCA2* polymorphisms by risk group

Risk group	*TP53* polymorphism (genotype)	*BRCA2* polymorphisms (genotype)		
	R72P (RR/RP/PP)	203 G>A (GG/GA/AA)	N372H (NN/NH/HH)	K1132K (KK/Kk/kk[a])
Low risk group (Beijing)				
Male, 56; female, 45	0.19/0.64/0.16[b]	0.40/0.48/0.11	0.50/0.50/0.00[b]	0.38/0.49/0.14
High risk group (Yangcheng)				
Male, 79; female, 51	0.29/0.52/0.18	0.36/0.55/0.10	0.58/0.34/0.08	0.37/0.52/0.12
ESCC cases				
Male, 74; female, 46	0.24/0.50/0.27	0.22/0.67/0.12[b]	0.58/0.33/0.10	0.37/0.47/0.16

[a] K is the wildtype allele and k is the variant allele.
[b] Not in Hardy–Weinberg equilibrium.

S114)0S) of *BRCA2* were identified. Three of the alterations were rare (1 each for R118H, C351S, and P3300S) and were not analyzed further.

3.1. Distribution of TP53 polymorphism R72P in low and high risk groups and ESCC

Genotype distributions for R72P were in HWE for all risk groups except the low risk group (Table 3), where the frequencies of genotypes RR (Arg/Arg), RP (Arg/Pro), and PP (Pro/Pro) were 0.19, 0.64, and 0.16, respectively, and the numbers of individuals with genotypes RR and PP were less than expected (HWE $\chi^2_{2\text{d.f.}} = 8.56$, $P = 0.014$).

The overall difference for the risk groups by genotypes (3×3 contingency table $\chi^2_{4\text{d.f.}} = 8.37$, $P = 0.079$), was due largely to the difference in distribution of genotypes between the low risk group and the ESCC group (2×3 contingency table $\chi^2_{2\text{d.f.}} = 5.47$, $P = 0.065$; Table 3). We did not find statistical evidence for a linear change in genotype frequency with increasing risk group ($\chi^2_{1\text{d.f.}} = 0.66$, P for trend $= 0.42$ for linear term in the proportional odds regression model). There was no significant difference between the high risk and the ESCC groups.

3.2. Distribution of three polymorphisms of BRCA2 in low and high risk groups and ESCC

The distributions of genotype frequencies of three *BRCA2* polymorphisms (203G>A, N372H, and K1132K) are shown for each of the three risk groups in Table 3. The genotype distributions of the three variants of *BRCA2* fit HWE for all risk groups except N372H in the low risk group and 203G>A in the ESCC group. Fewer than expected (i.e. zero) homozygous variants (HH) at N372H were observed in the low risk group (HWE $\chi^2_{2\text{d.f.}} = 10.93$, $P < 0.01$). At polymorphism 203G>A, there were fewer of both homozygote groups (GG and AA) than expected in the ESCC group (HWE $\chi^2_{2\text{d.f.}} = 14.43$, $P < 0.001$).

For 203G>A, there was an overall difference in the distribution of genotypes by risk groups ($\chi^2_{2\text{d.f.}} = 10.30$, $P = 0.036$) due to the distributional differences in genotypes observed between the ESCC group and the high risk ($\chi^2_{2\text{d.f.}} = 6.12$, $P = 0.047$) and low risk ($\chi^2_{2\text{d.f.}} = 9.20$, $P = 0.010$) groups. This, in turn, was attributed primarily to the low prevalence of the homozygous wildtype genotype (GG) in the ESCC group (0.22) compared to either the high risk group (0.36) or the low risk group (0.40). Using the proportional odds regression model we found a linear trend for increased frequency of genotypes with the A allele with increasing risk group ($\chi^2_{1\text{d.f.}} = 5.59$, P for trend $= 0.018$). As we moved from the lowest to highest risk groups, there was a monotonic decrease in the frequency of the homozygous wildtype genotype (0.40 to 0.36 to 0.22 for low risk to high risk to ESCC) and a parallel increase in the frequency of the heterozygote genotype (0.48 to 0.55 to 0.67).

For N372H, there was also an overall difference in the distribution of genotypes by risk groups ($\chi^2_{4\text{d.f.}} = 14.55$, $P = 0.006$) driven by distributional differences in genotypes in the low risk group compared to both the high risk group ($\chi^2_{2\text{d.f.}} = 12.50$, $P = 0.002$) and the ESCC group ($\chi^2_{2\text{d.f.}} = 13.55$, $P = 0.001$). These findings were the result of there being zero homozygous variants (HH) in the low risk group compared to either the high risk group (0.08) or the ESCC group (0.10). There was no difference between the high risk group and ESCC cases. Unlike 203 G>A, there was no real appearance of a linear relationship between genotype frequency and risk group ($\chi^2_{1\text{d.f.}} = 0.99$, P for trend $= 0.32$).

In contrast to the other genotypes examined, there was no overall difference in the distribution of genotypes for K1132K by risk groups ($\chi^2_{2\text{d.f.}} = 1.09$, $P = 0.897$), and more detailed comparisons were not pursued.

No differences in the distribution of genotypes by gender were seen when examined within risk groups or overall (data not shown), so only data for combined genders are presented here.

3.3. Associations between TP53 and BRCA2 polymorphisms in low and high risk groups and ESCC

The distributions of genotypes (combined across our three risk groups) for each of the three *BRCA2* polymorphisms (203G>A, N372H, and K1132K) in *BRCA2* were compared

separately to the genotype distribution of R72P in *TP53* in 3 × 3 contingency tables, but no statistically significant associations were found between polymorphic variants in these two genes in our three risk groups (data not shown).

4. Discussion

The present study was an initial investigation of the distribution of genotype frequencies for selected polymorphisms within the *TP53* and *BRCA2* genes in groups of northern Chinese with varying degrees of risk for ESCC. While we found potential associations for three genotypes and risk of ESCC, methodologic considerations dictate conservative interpretation of these data, and the results will require further study in other populations using more traditional control population selection procedures, more covariate information, and larger number of subjects before conclusions can be drawn regarding the role of these polymorphisms in risk assessment for ESCC.

The distribution of R72P in *TP53* has been reported to vary by ethnic group [17], with frequencies of the variant Pro allele ranging from a low of 0.17 in Swedish Saamiss [21] to a high of 0.63 in both African blacks [22] and northern Chinese from Linxian [20]. Compared to other studies in Chinese subjects, the healthy subjects from two different populations tested here showed a higher frequency of heterozygotes (0.52–0.64) than reported in Taiwanese (0.43–0.49) [16,19,22] or residents of Linxian (0.42) [20]. Further, we saw a lower frequency of both homozygous wild-type (0.19–0.29) and homozygous variant (0.16–0.18) genotypes than in Taiwanese (0.31–0.37 for RR and 0.17–0.23 for PP, respectively), while Linxian residents had lower RR (0.16) but much higher PP (0.42) genotype frequencies. The resulting frequencies of the Pro allele were 0.45–0.48 in these healthy populations, slightly higher than reported in Taiwanese (0.40–0.44), but substantially lower than in Linxian (0.63). Interestingly, the frequency of the Pro allele in ESCC cases in our study (0.52), ESCC cases from Taiwan (0.52), and ESCC cases from Linxian (0.55) were essentially the same. However, the findings (and conclusions) from each of these three studies differed: the Pro allele was associated with increased risk in Taiwan and was suggested as a risk factor in our study, but appeared to be protective in Linxian. These nonconcordant results are due to the wide variation in the Pro allele frequency in the different comparison groups used, as noted above, in addition to imprecision associated with small numbers, especially for the Linxian study. While the association of the Pro allele to ESCC risk may truly vary in different populations, evaluation in other subjects and populations with unbiased controls will be needed to sort out this relationship in the future. Although the functional significance of R72P in *TP53* is unknown, one recent study found that RR induced apoptosis with faster kinetics, was a better inducer of transcription, and suppressed transformation more efficiently than PP [23]. The association of this variant with lung, breast, and other cancer risks has been studied by several groups with inconsistent results [22,24–28]. Recently, allelic loss at polymorphism R72P in ESCC or in HPV-associated ESCC has been reported [8,29]. Although these changes may not cause tumors, they most likely contribute to tumor progression, especially allelic loss of R72P in HPV-associated esophageal cancer [29].

The frequency of genotype GG of 203G>A in *BRCA2* decreased in monotonic fashion from low risk (0.40) to high risk (0.36) to the ESCC patient group (0.22), while the heterozygote genotype increased monotonically across the same groups (0.48 to 0.55 to 0.67), suggesting an association for the 203G>A polymorphisms and risk of ESCC. Although the polymorphism 203G>A is in a non-coding region of *BRCA2*, a recent study showed that only chromosomes carrying G at the 203 position of the 5'UTR contributed normal mRNA, and that chromosomes carrying A (adenine) altered the reading frame and did not contribute to the production of normal *BRCA2* protein [30]. In the same study, the authors also reported that RNA analysis from a breast cancer patient with the *BRCA2* mutation IVS7 + 2T > G showed that the productive message was produced from only one chromosome, suggesting that intronic or intron–exon junction variants may contribute to the presence of normal RNA [30]. In a previous study, 10 of 51 ESCC with genotype GA had lost an allele in the tumor, and in all 10 cases it was the G allele that was lost, a finding consistent with a role for the A allele in ESCC susceptibility [15].

Several studies have demonstrated that *BRCA2* specifically interacts with histone acetyltransferase P/CAF, which possesses the histone acetyltransferase activity. This interaction requires residues 290–453 in the N-terminus of *BRCA2* where N372H is located [31]. It is not clear, however, whether the N372H substitution affects this binding. The frequencies of the Asn (0.75) and His alleles (0.25) at N372H in both of the healthy populations studied were similar to those reported in Caucasians (0.22–0.29 for His allele) [18]. The genotype distribution for N372H in our low risk group was not in HWE, a result also reported by Healey et al. [18]. Although we found significant differences in the distribution of N372H genotypes consistent with increased risk for persons with the HH genotype, these differences appear to reflect the low prevalence of HH in our low risk control group due to lack of HWE, thus we are reluctant to suggest that this reflects a true association with risk for ESCC based on these data alone. Of note, we found no differences in the distribution of genotypes by gender within risk groups or overall in our study, while Healey et al. found both lower than expected homozygotes in normal females and excess homozygotes in control males [18]. Differences between studies may reflect the different ethnic groups studied or may simply reflect the relatively small sample size in our study.

In conclusion, distributions of genotype and allele frequency of four common variants of *TP53* and *BRCA2* were assessed and compared in northern Chinese subjects at

varying degrees of risk, including both healthy subjects and ESCC patients. Differences in R72P (*TP53*) and N372H (*BRCA2*) were identified but appear to be due to especially low prevalences in our low risk control group, likely a reflection of lack of HWE. In contrast, the difference in 203G>A in *BRCA2* was due to low prevalence of GG in ESCC patients and is consistent with the decrease in transcript associated with this genotype, suggesting an association with disease. Overall, these data suggest that the 203G>A polymorphism in *BRCA2* may be associated with risk of ESCC, but replication in a more rigorously controlled study population is necessary to confirm this finding.

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